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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N.Y. 10022
(212) 421-8885

RECEIVED
JUN 13 1975

Date: May 23, 1975

Application for Research Grant
(Use extra pages as needed)

1. Principal Investigator (give title and degrees): Dr. Gad Feinstein, Senior Lecturer in Biochemistry. B.Sc., M.Sc., Ph.D.

2. Institution & address: Department of Biochemistry, The George S.Wise Center of Life Sciences, Tel-Aviv University, Ramat-Aviv, Tel-Aviv, Israel.

3. Department(s) where research will be done or collaboration provided:
   A. Research will be done at the Biochemistry Department, Tel-Aviv University, Ramat-Aviv, Tel-Aviv, Israel.
   B. Unpaid consultation will be provided by Dr. Aaron Janoff, Dept. of Pathology, SUNY at Stony Brook, Stony Brook, N.Y.

4. Short title of study:
Studies on Peptide Bond Specificities, Active Site and Inhibition of Human Leucocyte Proteases Which are Implicated in the Pathogenesis of Pulmonary Emphysema

5. Proposed starting date: January 1, 1976

6. Estimated time to complete: December 31, 1977

7. Brief description of specific research aims:
   A. Identification of the amino acid residues that are involved in the binding and catalytic active sites of the two leucocyte proteases, elastase and chymotrypsin-like protease.
   B. Characterization of the peptide bond specificities of these two proteases.
   C. Studies on the degradation of $^{35}SO_4$-labelled tracheobronchial cartilage by purified leucocyte proteases.
   D. Inhibition and inactivation studies of the elastase and chymotrypsin-like protease by naturally occurring inhibitors and synthetic inactivators.

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8. Brief statement of working hypothesis:

See attached paper.

9. Details of experimental design and procedures (append extra pages as necessary)

See attached paper.

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8. Several lines of investigation have focussed attention on leucocyte proteases, expecially elastase, as mediators of lung destruction in pulmonary emphysema associated with α_1-antitrypsin deficiency (Eriksson [1965]; Mittman [1972]). Levels of leucocyte elastase appear to be related to clinical prognosis in individuals with this disease (Galdston et al. [1973]; Rodriguez et al. [1975]). Elastolytic titer is positively correlated with the severity of lung lesions in animal models of emphysema induced by endotracheal protease instillation (Snider et al. [1974]; Blackwood et al. [1973]). Anatomic emphysema can be produced in dogs by aerosols of human and dog leucocyte homogenates, and the lesion severity is positively correlated with the titer of neutral protease activity in the homogenate (Mass et al. [1972]). Both proteases, leucocyte elastase and chymotrypsin-like protease, were reported (Lieberman and Gawad [1971]) to be capable of degrading human lung tissue. This led to the hypothesis (Lieberman and Gawad [1971]; Lieberman [1973]) that uninhibited proteolytic activities of leucocytes and macrophages result in pathogenesis of pulmonary emphysema.

The purification of human leucocyte elastase was accomplished in this laboratory (Janoff [1973]). More recently, larger scale purification of human leucocyte elastase was reported by us (Feinstein and Janoff [1975a]). The elastase was further characterized with respect to its amino acid composition, molecular weight, synthetic substrate specificity and antigenic properties (including preparation of a monospecific anti-serum). A second human leucocyte protease, chymotrypsin-like neutral protease, was purified by this laboratory (Feinstein and Janoff [1975b]). Some of the physico-chemical properties of the chymotrypsin-like protease were studied including its inhibition by serum protein inhibitors and synthetic inactivators. This latter protease was reported recently, like the granulocyte elastase, to be capable of degrading a natural protein substrate, articular cartilage proteoglycan (Malemud and Janoff [1975]; Feinstein et al. [1975]).

Our working hypothesis is that leucocytic neutral proteases, especially the elastase and the chymotrypsin-like protease, play a significant role in the pathogenesis of pulmonary emphysema. Therefore, proposed studies on peptide-bond specificities, active centers and inhibition of these enzymes could provide important information necessary for their control in individuals with chronic obstructive lung diseases.

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List of References for Item No. 8

Blackwood, C.E., Hosannah, Y., Preman, E., Keller, S. and Mandel, I., 1973. Experimental Emphysema in Rats: Elastolytic Titer of Inducing Enzyme as Determinant of the Response, Proc. Soc. Expt. Biol. Med. 144, 450-454.

Erickksson, S. 1965. Studies in α1-Antitrypsin Deficiency. Acta. Med. Scand. 177, Suppl. 432, 1-85.

Feinstein, G. and Janoff, A., 1975a. A Rapid Method for Purification of Human Granulocyte Cationic Neutral Proteases: Purification and Further Characterization of Human Granulocyte Elastase. Biochim. Biophys. Acta., submitted for publication.

Feinstein, G. and Janoff, A., 1975b. A Rapid Method for Purification of Human Granulocyte Cationic Neutral Proteases: Purification and Characterization of Human Granulocyte Chymotrypsin-Like Protease. Biochim. Biophys. Acta., submitted for publication.

Feinstein, G. Malemud, C.J. and Janoff, A., 1975. The Degradation of Articular Cartilage $^{35}SO_4$ by Purified Human Granulocyte Proteases. Manuscript in preparation.

Galdston, M., Janoff, A. and Davis, A.L., 1973. Familial Variation of Leukocyte Lysosomal Protease and Serum α1-Antitrypsin as Determinants in Chronic Obstructive Pulmonary Disease. Am. Rev. Resp. Dis. 107, 718-727.

Janoff, A. 1973., Purification of Human Granulocyte Elastase by Affinity Chromatography. Lab Invest. 29, 458-464.

Lieberman, J. and Gawad, M.A., 1971. Inhibitors and Activators Leukocytic Proteases in Purulent Sputum. Digestion of Human Lung and Inhibition by Alpha$_1$-Antitrypsin. J. Lab. Clin. Med. 77, 713-727.

Lieberman, J., 1973. Involvement of Leukocytic Proteases in Emphysema and Antitrypsin Deficiency. Arch. Environ. Health 27, 196-200.

Malemud, C.J. and Janoff, A., 1975. Identification of Neutral Proteases in Human Neutrophile Granulocytes which Degrade Articular Cartilage Proteoglycans. Arthritis and Rheum., in press.

Mass, B., Ikeda, T., Meranze, D.R., Weinbaum, G. and Kimbel, P., 1972. Induction of Experimental Emphysema. Cellular and Species Specificity. Am. Rev. Resp. Dis. 106, 384-391.

Mittman, C., Ed. "Pulmonary Emphysema and Proteolysis". Academic Press. New York and London. 1972.

Rodriguez, J.R., Seals, J.E., Radin, A., Lin, J.S., Mandel, I. and Turino, G.M., 1975. The Role of Leucocyte Lysosomal Elastase in the Pathogenesis of Obstructive Lung Disease. Clin. Res. 23, 349A.

Snider, G.L., Hayes, J.A., Franzblau, C., Kagan, H.M., Stone, P.S. and Korthy, A.L., 1974. Relationship Between Elastolytic Activity and Experimental Emphysema-Inducing Properties of Papain Preparations. Am. Rev. Respt. Dis. 110, 254-262.

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9. Planned Experimental Procedures

A. Identification of the amino acid residues that are involved in the binding and catalytic active sites of the two proteases, elastase and chymotrypsin-like protease. Purified enzymes will be labeled by use of radioactive-labeled specific active-site directed reagents. The labeled enzymes will be degraded by proteolytic enzymes, trypsin and subtilisin, into peptides which will be separated from each other by various procedures like ion-exchanges chromatography, molecular sieving and electrophoresis. The amino acid sequencing of the purified radioactively labeled peptides will give the sequence of amino acids in the active sites of the leucocyte proteases.

B. Characterization of the peptide-bond specificities of these two proteases. Proteins with well known amino acid sequences will be subjected to proteolysis by the purified leucocyte proteases. The peptides obtained will be separated from each other. From the amino acid composition of the peptides it will become evident which peptide bonds in proteins are susceptible to hydrolysis by the leucocyte proteases. In addition a series of small peptides will be tested for their susceptibility to hydrolysis by the proteases. The elucidation of substrate specificity of the enzymes will help to design synthetic inactivators of the proteases.

C. Studies on the degradation of $^{35}SO_4$-labeled tracheobronchial cartilage by purified leucocyte proteases. Lung $^{35}SO_4$-labeled cartilage will be prepared by injection of $Na_2{}^{35}SO_4$ to rabbits which will be sacrificed after 18-24 hours. Tracheal cartilage rings and bronchial cartilage plates will be removed, cleaned and used as substrate for the action of the proteases. The release of soluble $^{35}SO_4$ from the lung tissue will indicate that proteolysis has taken place. The kinetics of the proteolysis and its inhibition by various inhibitors will be studied.

D. Inhibition and inactivation studies. These will include the testing of the capacities of naturally occurring inhibitors from a variety of sources to inhibit the leucocyte proteases. Synthetic inactivators will also be tested for their capacities to specifically inactivate these proteases. This approach will be followed with the purpose of developing inhibitors or inactivators with potential prophylactic application in subclinical stages of the disease.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location): All the departmental facilities of the the department of biochemistry at Tel-Aviv University will be available for this research. These include: Analytical Ultracentrifuge, Amino Acid Analyzer, High Resolution Spectrophotometers (Cary 14 etc.), High Voltage Electrophoresis, Preparative Ultracentrifuges, Potentiometric Titrator, Spectroflourometer, Gas Liquid Chromatography, etc.

[illegible] of radioactive-labeled specific active-site directed [illegible] labeled enzymes will be degraded by proteolytic enzymes, trypsin and subtilisin, into peptides which will be separated from each other [illegible] various procedures like ion-exchanger chromatography, molecular sieving and electrophoresis. The amino acid sequencing of the purified radioactively labeled peptides will give the sequence of amino acids in the active sites of the leucocyte proteases.

B. Characterization of the peptide-bond specificities of these two proteases. Proteins with well known amino acid sequences will be [illegible] peptides [illegible] will be separated from each other [illegible] amino acid composition of the peptides it will become evident which peptide [illegible]

11. Additional facilities required: Recorder, U.V. monitor and High pressure pump.

C. Studies on the degradation of [illegible] labeled trachea-bronchial cartilage [illegible] prepared by injection of $Na_2{}^{35}SO_4$ to rabbits which will be sacrificed [illegible] plates will be removed, cleaned and used as substrate for the action [illegible] proteolysis and its inhibition by various inhibitors will be studied.

D. [illegible]

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Dr. Gad Feinstein	33	REDACTED
Technical		
Technician (To be recruited)	100	REDACTED
	Sub-Total for A	REDACTED

B. Consumable supplies (by major categories)

Chemical Reagents	- 2500
Ion Exchangers	- 1000
Blood	- 1000
Enzymes and Proteins	- 1000
Glassware	- 1500
Animals	- 1000
	Sub-Total for B REDACTED

C. Other expenses (itemize)

Manuscript Typing and Reprints	- 250
Indexing Service	- 200
Books	- 150
Domestic Travel in Israel	- 300
	Sub-Total for C 900
	Running Total of A + B + C 17,985.-

D. Permanent equipment (itemize)

Recorder	- 1400
UV Monitor	- 1500
High Pressure Pump	- 1300
	Sub-Total for D 4,200

E. Indirect costs (15% of A+B+C) E 2,698.-

Total request 24,883.-

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	9,000	1,000	--	2,700	20,700
Year 3	--	--	--	--	--	--

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Protein Inhibitors in Synovial Fluids of Patients with Joint Diseases	Israeli Ministry of Health	$4000	October 1, 1975

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

Mailing address for checks

Principal investigator

Typed Name Dr. Gad Feinstein

Signature Gad Feinst Date May 23, 1975

Telephone Israel 03 (Area Code) 416-111 (Number) 749 (Extension)

Responsible officer of institution

Typed Name I. Levanon

Title Director Research Authority

Signature I. Levanon Date

Telephone (Area Code) (Number) (Extension)

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CURRICULUM VITA

GAD FEINSTEIN, Ph.D.

Personal Data: Born: REDACTED Married: REDACTED

Citizenship: REDACTED Children: REDACTED

Education:

REDACTED Studies. The Hebrew University of Jerusalem, Israel. B.Sc. (1962) in Agriculture.

REDACTED Studies. The University of California, Davis. M.Sc. (1963) in Food Science.
Thesis: Chemical and Physical Properties of the Proteolytic Enzymes of Stem Bromelain.
Instructor - Prof. John R. Whitaker..

Ph.D. (1966) in Comparative Biochemistry.
Thesis: Chemistry of the Inhibitors of Proteolytic Enzymes and Studies of the Mechanism of Action.
Instructor - Prof. Robert E. Feeney.

REDACTED Post-graduate studies. Biology Department, Brookhaven National Laboratory, Upton, New York, with Dr. Elliot N. Shaw.
Subject: Enzyme Chemistry.

Experience:

1962-1966	Graduate Research Assistant, Department of Food Science, University of California, Davis, California.
1966-1968	Research Associate, Biology Department, Brookhaven National Laboratory, Upton, New York.
1968-1973	Lecturer, Biochemistry Department, Tel-Aviv University, Tel-Aviv, Israel.
1973-	Senior Lecturer in Biochemistry (Tenure).
1974-1975	On a Sabbatical Leave from Tel-Aviv University.
	Visiting Lecturer in Pathology, Pathology Department, Basic Health Sciences, State University of New York at Stony Brook, Stony Brook, New York.

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No. 13 Recent and Pertinent List of Publications

1. Gad Feinstein, Abraham Kupfer and Mordechai Sokolovsky.
N-Acetyl-(L-Ala)$_3$-p-Nitroanilide as a New Chromogenic Substrate for Elastase.
Biochem. Biophys. Res. Commun. 50, 1020-1026 (1973).

2. Gad Shtacher, Rachel Maayan and Gad Feinstein.
Proteinase Inhibitors in Human Synovial Fluid.
Biochem. Biophys. Acta 303, 138-147 (1973).

3. Emmanual Shapira, Nili Peylan-Ramu, Yoav Ben-Yoseph, Gad Feinstein and Mordechai Sokolovsky.
Specific Immunoassay for Quantitive Determination of Human Chymotrypsin in Intestinal Content.
Israel J. Med. Sci. 10, 1086-1091 (1974).

4. Gad Feinstein, Ronny Hoffstein, Joseph Koifman and Mordechai Sokolovsky.
Human Pancreatic Proteolytic Enzymes and Protein Inhibitors: Isolation and Molecular Properties.
Europ. J. Biochem. 43, 569-581 (1974).

5. Gad Feinstein, Ronny Hoffstein and Mordechai Sokolovsky.
Isolation of Human Pancreatic Inhibitor and Study of Its Interaction with Mammalian and Human Proteases.
Proteinase Inhibitors, Bayer-Symposium V, p. 199-212 (H. Fritz, H. Tschesche, L.J. Greene and E. Truscheit, Eds.). Springer-Verlag, Berlin. Heidelberg.New York. 1974.

6. Gad Feinstein and Aaron Janoff.
A Rapid Method for Purification of Human Granulocyte Cationic Neutral Proteases: Purification and Characterization of Human Granulocyte Chymotrypsin-Like Protease.
Biochim. Biophys. Acta., submitted for publication.

7. Gad Feinstein and Aaron Janoff.
A Rapid Method for Purification of Human Granulocyte Cationic Neutral Proteases: Purification and Further Characterization of Human Granulocyte Elastase.
Biochim. Biophys. Acta., submitted for publication.

8. Gad Feinstein, Charles J. Malemud and Aaron Janoff.
The Degradation of Articular Cartilage $^{35}SO_4$ by Purified Human Granulocyte Proteases.
Manuscript in preparation.

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CURRICULUM VITAE

(Date of preparation: 5/73)

AARON JANOFF

Born: REDACTED REDACTED

PROFESSIONAL APPOINTMENTS

February 1, 1971: Professor of Pathology, State University of New York at Stony Brook
1966-January 1971: Associate Professor, Department of Pathology, N.Y.U. School of Medicine
1961-1966 : Assistant Professor, N.Y.U. School of Medicine, Department of Pathology
1959-1961 : Instructor, N.Y.U. School of Medicine, Department of Pathology
1958-1959 : Research Associate, N.Y.U. School of Medicine, Department of Pathology
1953-1958 : Teaching Fellow, New York University, Department of Biology

ADMINISTRATIVE APPOINTMENTS - N.Y.U. SCHOOL OF MEDICINE

Executive Admissions Committee
Pass/Fail Grading System Committee
Graduate Advisor, Basic Science Program, Department of Pathology
Medical Student Advisor to First Year Class

ADMINISTRATIVE APPOINTMENTS - S.U.N.Y., STONY BROOK

Director, Graduate Program in Experimental Pathology
Graduate Studies Committee, Graduate Council, Admissions Committee (Medici
Appointments, Promotions and Tenure Standing Committe

SOCIETY MEMBERSHIPS

REDACTED

REDACTED

HONORS, AWARDS

Phi Beta Kappa
A.B., cum laude
NSF Predoctoral Fellow, 1956-1958
USPHS Career Development Awardee, 1962-1971

TEACHING EXPERIENCE - N.Y.U. SCHOOL OF MEDICINE

"General Pathology" (Section: Inflammation and Infectious Disease)
"Subcellular and Molecular Pathology" (Section: Lysosomes)
"Biology of Cancer" (Section: Proteases and Co-Carcinogenesis)
Pathology Graduate Students Journal Club (Preceptor)

-Health Sciences Center, SUNY Stony Brook

General Pathology for Medical Students
General Pathology for Nurses, Physicians Associates and Allied Health Professionals
Lysosomes; Structure and Function of Biomembranes; Carcinogenesis (Grad. Cours

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INVITED SPEAKER - INTERNATIONAL MEETINGS

Vth International Congress on Angiology, Paris, France, 1964
Vth International Symposium on Immunopathology, Punta Ala, Italy, 19
Symposium on Pulmonary Emphysema and Proteolysis, City of Hope National Medical Center, Pasadena, California, 1971
IInd International Symposium on the Biochemistry of the Acute Aller Reactions, Brook Lodge, Michigan, 1971
Symposium on Neutrophil Proteases as Mediators of Tissue Injury (Chairman), Atlantic City, New Jersey, 1972
Gordon Conference on Lysosomes, Andover, New Hampshire, 1972
The New York Academy of Sciences (Biochemistry Section), 1972
NY Academy of Sciences Symposium on Rheumatoid Arthritis (Session Chairman) 1974
Cold Spring Harbor Symposium on Proteases and Biological Growth Control 1974

GOVERNMENT SERVICE

Pulmonary Diseases Advisory Committee (National Heart & Lung Institute) 1974-1977

RESEARCH INTERESTS

Purification of Human Leukocyte Proteases; Role of Leukocyte Proteases in the Mediation of Pulmonary Emphysema and Rheumatoid Arthritis; Synthetic Inhibitors of Leukocyte Proteases; Bacterial Degrading Functions of Human Leukocyte Protease

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List of References (Dr. A. Janoff) for Item No. 13

1) Janoff, A. Human Granulocyte Elastase: Further Delineation of its Role in Connective Tissue Damage. Am. J. Path. 68: 579, 1972.

2) Janoff, A. Neutrophil Proteases in Inflammation. Am. Rev. Med. 23: 177, 1972.

3) Galdston, M., Janoff, A. and Davies, A. Levels of Leukocyte Lysosomal Elastase and Serum Alpha 1-Antitrypsin as Determinants in the Expression of Chronic Obstructive Pulmonary Disease. Am. Rev. Resp. Dis. 107: 718, 1973.

4) Janoff, A. Purification of Human Granulocyte Elastase by Affinity Chromatography. Lab. Invest. 29:458, 1973.

5) Janoff, A., Blondin, J. Sandhaus, R.A., Mosser, A. and Malemud, C. Human Neutrophil Elastase: In Vitro Effects on Natural Substrates Suggest Important Physiological and Pathological Actions in: Cold Spring Harbor Symposium on Proteases and Biological Growth Control (eds: E. Reich, D. Rifkin and E. Shaw) Cold Spring Harbor Press, Cold Spring Harbor, NY, in press.

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